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Simultaneous Quantitation of Five Triazole Anti-fungal Agents by Paper Spray-Mass Spectrometry

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Abstract

Introduction: Invasive fungal disease is a life-threatening condition that can be challenging to treat due to pathogen resistance, drug toxicity, and therapeutic failure secondary to suboptimal drug concentrations. Frequent therapeutic drug monitoring (TDM) is required for some anti-fungal agents to overcome these issues. Unfortunately, TDM at the institutional level is difficult, and samples are often sent to a commercial reference laboratory for analysis. To address this gap, the first paper spray-mass spectrometry assay for the simultaneous quantitation of five triazoles was developed.

Methods: Calibration curves for fluconazole, posaconazole, itraconazole, hydroxyitraconazole, and voriconazole were created utilizing plasma-based calibrants and four stable isotopic internal standards. No sample preparation was needed. Plasma samples were spotted on a paper substrate in pre-manufactured plastic cartridges, and the dried plasma spots were analyzed directly utilizing paper spray-mass spectrometry (paper spray MS/MS). All experiments were performed on a Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer.

Results: The calibration curves for the five anti-fungal agents showed good linearity (R^2 = 0.98 – 1.00). The measured assay ranges (LLOQ – ULOQ) for fluconazole, posaconazole, itraconazole, hydroxyitraconazole, and voriconazole were 0.5 – 50 μg/mL, 0.1 – 10 μg/mL, 0.1 – 10 μg/mL, 0.1 – 10 μg/mL, and 0.1 – 10 μg/mL, respectively. The inter- and intra-day accuracy and precision were less than 25% over the respective ranges.

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Conclusion: We developed the first rapid paper spray MS/MS assay for simultaneous quantitation of five triazole anti-fungal agents in plasma. The method may be a powerful tool for near point-of-care TDM aimed at improving patient care by reducing turnaround time and for use in clinical research.

Keywords

anti-fungal; paper spray MS/MS; ambient ionization; quantitative; dried matrix spots; separation

Introduction:

Fungal disease is a significant clinical and economic burden in the healthcare system, and invasive fungal disease is a leading cause of morbidity and mortality in critically-ill and immunocompromised patient populations (1–4). Fortunately, the development of anti-fungal agents has dramatically improved clinical outcomes (5, 6). Despite these advancements, clinicians and pharmacists struggle with dosing of anti-fungal agents due to altered host pharmacokinetics, drug resistance, and risk of therapeutic failure at suboptimal concentrations (2, 7–9). Adverse effects are frequently observed, including hepatotoxicity and neurological effects (7, 8, 10). These issues, as well as unique anti-fungal pharmacokinetics and pharmacodynamics, have pushed the need for therapeutic drug monitoring (TDM) of certain agents (5, 7, 8).

There are three main classes of systemic anti-fungal agents in clinical use: polyenes, triazoles, and echinocandins. Triazoles are particularly attractive to clinicians as they have broad anti-fungal activity and can often be taken orally (6, 10–13). Most mold-active triazoles (itraconazole, voriconazole, and posaconazole) require frequent TDM for efficacy and safety (7, 8, 10–12, 14, 15).

Many assays utilizing high performance liquid chromatography-mass spectrometry (HPLC-MS) for the quantification of triazoles have been reported in the literature (16–20), and several centralized reference laboratories offer their own methods for clinical use. MS-based approaches are attractive due to their high sensitivity and specificity, lack of interference, multiplex capability, high throughput, and low reagent cost (21, 22). Nevertheless, logistics, technical expertise, and sample preparation (solid-phase extraction, liquid-liquid extraction, or protein precipitation) often hinder implementation at the institutional level, and patient samples must be shipped to reference laboratories, which leads to long turnaround times and high costs (21, 23). Delays in result reporting can have a negative impact on patient care and clinical outcomes; therefore, new methods that can be utilized near the point of care are needed (7, 8).

Paper spray, an ambient ionization technique, is an ideal method for therapeutic monitoring as it allows for rapid analysis of complex biological samples without sample preparation or chromatography (24–27). A small sample volume (5 – 15 μL) is deposited directly onto a porous, triangular paper substrate and allowed to dry. Analysis is performed directly from the dried spot via application of a spray solvent and a high voltage while the sharp tip of the paper is in close proximity to the inlet to the mass spectrometry (28), where the ions enter the mass spectrometer for detection. A sample can typically be analyzed in 60 – 90 seconds

(25). Paper spray MS/MS methods for fast qualitative and quantitative analysis of both small and large molecules from a variety of biological and environmental matrices have been reported, and it has shown potential for TDM (29–33). Paper spray MS has several advantages over LC-MS/MS methods, including easy sample preparation, lower solvent consumption, less expertise, and faster turnaround times (27, 29–32).

In this paper, we present the first validated paper spray MS/MS method for the simultaneous quantification of five anti-fungal drugs in plasma samples. Remnant patient clinical samples collected from the Indiana University Health Pathology Laboratory (Indianapolis, IN, USA) were analyzed by paper spray MS/MS. The results obtained by paper spray MS/MS were compared to measured levels obtained by a reference laboratory via LC-MS/MS (Mayo Clinic Laboratories, Rochester, MN, USA).

IRB Approval:

The study was reviewed and approved by the Indiana University - Purdue University Indianapolis Institutional Review Board.

Materials and Methods:

Materials and Reagents:

Analytical - grade methanol, water, acetonitrile, acetone, and ammonium acetate were purchased from Fisher Scientific (Pittsburg, PA, USA). Acetic acid, itraconazole, posaconazole, fluconazole, voriconazole, itraconazole-D4, fluconazole-13C3, and voriconazole-D3 were purchased from Sigma Aldrich (St. Louis, MO, USA). Hydroxyitraconazole and hydroxyitraconazole-D5 were purchased from Fitzgerald Industries International (Acton, MA, USA) and Toronto Research Chemicals Incorporated (Ontario, Canada). The external quality control was purchased from UTAK Laboratories (Valencia, CA, USA). Pre-made plastic cartridges were purchased from Prosolia, Inc. (Indianapolis, IN, USA). Whatman grade 31ET chromatography paper was purchased from Fisher Scientific (Lenexa, KS, USA).

Sample Preparation:

Stock solutions were prepared in *N,N*-dimethylformamide (DMF) at the following concentrations: fluconazole (6.0 mg/mL), voriconazole (1.0 mg/mL), hydroxyitraconazole (1.0 mg/mL), posaconazole (2.0 mg/mL), and itraconazole (2.0 mg/mL). Five spiking solutions (SS1 – 5), each containing all five analytes, were then prepared in 95:5 methanol/water with 0.01% acetic acid. The concentrations for itraconazole, hydroxyitraconazole, posaconazole, and voriconazole were 200.0 μg/mL (SS1), 60.0 μg/mL (SS2), 20.0 μg/mL (SS3), 6.0 μg/mL (SS4), and 2.0 μg/mL (SS5). Fluconazole concentrations were 1000.0 μg/mL (SS1), 300.0 μg/mL (SS2), 100.0 μg/mL (SS3), 30.0 μg/mL (SS4), and 10.0 μg/mL (SS5). Stock and spiking solutions were stored at −20°C. Plasma calibrants were prepared by spiking a 100 μL aliquot of plasma with 5 μL of the corresponding spiking solution (SS1 – SS5) to make final plasma concentrations of 10.0 μg/mL, 3.0 μg/mL, 1.0 μg/mL, 0.3 μg/mL, and 0.1 μg/mL of itraconazole, hydroxyitraconazole, posaconazole, and

voriconazole. Fluconazole final plasma concentrations were 50.0 μg/mL, 15.0 μg/mL, 5.0 μg/mL, 1.5 μg/mL, and 0.5 μg/mL.

Internal quality controls (QC) were prepared similarly to the calibrants. Internal QCs at four different concentrations (LLOQ, low, medium, and high) were utilized in this experiment. Four spiking solutions (QCS1 – 4) were prepared in 95:5 methanol/water with 0.01% acetic acid at the following concentrations: 200.0 μg/mL (QCS1), 20.0 μg/mL (QCS2), 6.0 μg/mL (QCS3), and 2.0 μg/mL (QCS4) for itraconazole, hydroxyitraconazole, posaconazole, and voriconazole. Fluconazole concentrations were 1000.0 μg/mL (QCS1), 100.0 μg/mL (QCS2), 30.0 μg/mL (QCS3), and 10.0 μg/mL (QCS4). QC samples were prepared by spiking a 100 μL aliquot of plasma with 5 μL of the corresponding spiking solution (QCS1 – 4) to make final concentrations in plasma of 10.0 μg/mL, 1.0 μg/mL, 0.3 μg/mL, and 0.1 μg/mL of itraconazole, hydroxyitraconazole, posaconazole, and voriconazole. Final plasma concentrations for fluconazole were 50.0 μg/mL, 5.0 μg/mL, 1.5 μg/mL, and 0.5 μg/mL. The external QCs were prepared according to the manufacturer's instructions. Concentrations for fluconazole, itraconazole, hydroxyitraconazole, voriconazole, and posaconazole in the external QC were reported as: 18.0 μg/mL, 4.94 μg/mL, 2.87 μg/mL, 3.47 μg/mL and 5.09 μg/mL.

The internal standard solution was prepared in 50:50 methanol/50 mM ammonium acetate. The final concentrations of the stable isotopically-labelled analogs in the internal standard solution were: voriconazole-D3 (1.0 μg/mL), itraconazole-D4 (4.0 μg/mL), hydroxyitraconazole-D5 (2.0 μg/mL) and fluconazole-13C3 (10.0 μg/mL). A 10 μL aliquot of the internal standard solution was added to all plasma samples with the exception of the double blank plasma samples. Each plasma sample was vortexed for 10 seconds to thoroughly mix. Eight microliters of each sample was then spotted onto the paper substrate contained within the paper spray cartridge. Cartridges were covered to protect samples from ambient light and were allowed to dry for one hour at room temperature prior to analysis. Of note, the internal standard solution was found to be stable for four days when stored at −20°C.

Remnant patient clinical samples were gathered from the Indiana University Health pathology laboratory where they were stored at −20°C until retrieval. The clinical samples were de-identified, transported on dry ice, and stored at −80°C until use. Patient plasma aliquots (100 μL) were spiked with 10 μL of internal standard solution and spotted on the paper spray cartridges as described above for calibrants and QCs.

Paper Spray Mass Spectrometry Assay Development:

Paper spray was performed utilizing plastic cartridges containing Whatman grade 31ET chromatography paper. An automated paper spray source, the Velox 360 (Prosolia, Inc., Indianapolis, USA), was coupled to a Thermo Fisher Scientific TSQ Vantage triple quadrupole mass spectrometer (San Jose, CA, USA). Optimized mass spectrometry parameters were as follows: 300°C capillary temperature, 4200V spray voltage, positive ion mode, and no sheath or auxiliary gas. The instrument was operated in selected reaction monitoring (SRM) mode with a dwell time of 0.1 seconds. SRM transitions and optimized instrument parameters for the five triazoles and the stable isotopic internal standards are

shown in Table 1. The automated Velox 360 source parameters were: Cartridge dispense: Pump B; Number of pump B dispenses: 9; Dispense volume: 90 μL; Pump B dispense delay: 0 seconds.

Method Validation:

Each analytical run consisted of calibrants, internal QCs, external QC, remnant patient clinical samples, blanks with internal standard, and double blanks without internal standard. Calibration curves were run in duplicate, one at the beginning and one at the end of each experimental run. The number of external and internal QCs for each analytical run was ≥5% of remnant clinical samples as recommended by FDA guidelines (34). Two or more replicates of each QC (four internal QCs, one external QC) were performed per analytical run. More than 67% of all QCs and 50% of QCs at each concentration level had to meet the acceptance criterion of a difference of ≤25% of the known nominal concentration for the analytical run to be considered valid. Plasma blanks with internal standard and plasma double blanks were used to assess carryover and blank signal. Remnant patient clinical samples were run in singlet during the validation and calculated values were compared to values determined by a validated LC-MS/MS-based method (Mayo Clinic Laboratories, Rochester, MN, USA). A total of 110 clinical samples were run: 66 voriconazole, 24 itraconazole/hydroxyitraconazole, and 20 posaconazole samples. Fluconazole patient clinical samples were unavailable at the time of validation.

Long-term Stability:

Remnant Clinical Sample Stability:

Due to the lengthy handling process of the clinical samples, degradation was studied in clinical samples over the course of 14 days. Ten samples for each drug were stored under three temperature conditions (−20°C, 4°C and 22°C) and were analyzed in duplicate after 0, 7, and 14 days. The samples were analyzed using the validated method and checked for degradation. For samples to be considered passing, differences in nominal concentration could not exceed 20%. Additionally, spiking solutions for the calibrants and QCs were evaluated for linearity, accuracy, and precision at the beginning of each day.

Assessment of Endogenous Interference:

Hemolysis was assessed in accordance with established protocols (35). Briefly, fresh drug-free whole blood was shaken vigorously and stored at −20°C for 30 minutes. Hemolyzed blood was spiked into blank plasma to create two test groups consisting of 0.5% (0.5 μL hemolyzed blood in 100 μL of blank plasma) and 2% (2 μL hemolyzed blood in 100 μL of blank plasma) hemolyzed plasma. A test group with 0% hemolysis was utilized as a control. Low concentration QCs utilizing plasma from the three test groups were prepared, and five replicates were analyzed as described above. To be considered negligible, the difference in nominal concentration between hemolyzed and non-hemolyzed samples had to be ≤25% for all analytes.

Drug-free icteric and lipidemic plasma samples were collected from the Indiana University Health pathology laboratory and stored at −20°C until use. QCs at the high and low levels

were prepared in five separate lipidemic samples and three separate icteric samples. QCs prepared in normal plasma was used as the control. Five replicates of each sample were analyzed as described above. To be considered negligible, lipidemic and icteric samples had to meet precision and accuracy acceptance criteria of ≤ 25% for all analytes.

Data and Statistical Analysis:

Data analysis was performed utilizing Tracefinder 3.3 (Thermo Fisher Scientific Inc., San Jose, CA, USA). The calibration curves were calculated using $1/x^2$ weighted linear least squares (36). All statistics were performed utilizing Minitab (Minitab Inc., State College, PA, USA) or Excel (Microsoft Corp., Redmond, WA, USA).

To further assess accuracy and systematic bias of the paper spray MS/MS assay, a comparison of the two methods was performed utilizing Passing-Bablok regression (37), which calculates a regression equation ($y = a + bx$) and the 95% CIs for the constant (a) and proportional bias (b). A Kolmogorov-Smirnov CUSUM test was performed to confirm the linearity of these values as assumed by the Passing-Bablok regression. A p value of <0.05 indicates a statistically significant deviation from linearity. Bland-Altman plots were utilized to further assess agreement and bias between measured results of the two methods (37).

Results:

Method Validation:

Validation was performed utilizing Food and Drug Administration (FDA) guidelines as a basic framework (34). The method was validated in terms of linearity, limit of detection (LOD), lower limit of quantification (LLOQ), accuracy (%bias), precision (%CV), matrix effects, carry-over, stability, and endogenous interference. Figure 1 shows an overlay of the calibration curves collected over seven different days for each analyte. The calibration curves had average coefficients of determination (R^2) of ≥0.99 with the exception of posaconazole (≥0.98). Posaconazole did not have a stable isotopically-labeled analog as its internal standard; deuterated hydroxyitraconazole was used as the internal standard instead. Table 2 shows the observed variation in the calibration curve slopes, which was <5% in all cases. The average calculated LODs and measured LLOQs are shown in Table 2. Table 3 shows the average intra and inter-day accuracy (%bias) and precision (%CV) for all analytes. These values were below the established acceptance criteria of ≤25% for both accuracy and precision values.

Stock and spiking solutions were evaluated for stability over the course of three months. No degradation was found for the stock solutions dissolved in DMF. Degradation was observed at 10 weeks in the neat spiking solutions stored at −20°C (data not shown). In addition, no significant degradation was found when analyzing remnant clinical patient samples kept at three different temperatures (−20°C, 4°C, and 22°C) over the course of two weeks. Of note, internal standard signal was found to decrease over time when the dried plasma spots were exposed to constant ambient light. Figure 2 shows itraconazole-D4 signal (**2A**) when the plasma spots were exposed to light over the course of 16 hours. Significant signal decrease occurred over the course of this experiment. This decrease in signal response was largely

eliminated by covering the samples (**2B**). This same trend was seen for the other three internal standards (data not shown), and similarly, the issue was resolved when shielding the spotted samples from ambient light.

Endogenous interference from two common metabolites, posaconazole N-β-D-glucuronide and voriconazole-N-oxide, was evaluated. Interference from posaconazole glucuronide on the posaconazole SRM channel was found to be 3%. Voriconazole-n-oxide was also evaluated, and the interference was negligible. In addition, the potential effects of hemolysis, icterus, and lipidemia were evaluated. All lipidemic and hemolyzed samples passed within the established acceptance criterion of ≤25%. All icteric samples passed within limits with the exception of posaconazole high QC samples, which indicates there could be a potential interference from bilirubin at high posaconazole concentrations.

Matrix effects were assessed utilizing a method developed by Matuszewski *et al.*, in which calibration curves were prepared in seven separate individual donor lots of plasma (38). Matrix effects were then assessed by determining the variation of the calibration slopes. The %CV of the slopes were 3% for fluconazole, 4% for itraconazole and voriconazole, and 10% for posaconazole. In addition, no carryover was noted during the course of the validation.

Reference Laboratory Cross-Validation:

Anti-fungal concentrations in remnant clinical samples were measured via the validated paper spray MS/MS method and compared to the values obtained via LC-MS/MS. The normality of the differences between the two methods were tested using a Kolmogorov-Smirnov CUSUM test, and all data were considered linear ($p = \geq 0.5450$). A Passing-Bablok regression analysis was performed to assess correlation between the paper spray MS/MS concentrations and the LC-MS/MS values (Figure 3). Regression equations and 95% CIs for the slope and intercept of each analyte (dashed lines) are displayed on each plot. High R^2 values (0.90 – 0.98) were obtained for all analytes, which indicated good linearity and correlation between the two methods. The individual data points were randomly distributed around the best fit regression line indicating no obvious trends in the data. When evaluating the 95% CIs around the regression line in relation to the line of equality (y = x line, where slope = 1 and intercept = 0), the regression lines for voriconazole and posaconazole results were not significantly different than equality. For both itraconazole and hydroxyitraconazole, the slope of 1 was outside the 95% CI, indicating a proportional negative bias of paper spray MS.

Bland-Altman plots depicting the relative difference between the two methods versus mean concentration are displayed in Figure 3. The mean bias of paper spray MS was −5% (1.96*SD: −30% to +21%) for voriconazole, −21% (1.96*SD: −40% to −1.2%) for itraconazole, −6% (1.96*SD: −28% to +16%) for hydroxyitraconazole, and −12% (1.96*SD: −37% to +13%) for posaconazole.

Discussion:

The first paper spray MS/MS method for the rapid simultaneous quantitation of five anti-fungal triazoles was developed and cross-validated with clinical samples. Overall, the

calibration curves showed good linearity (R^2 = 0.94 – 1.00) for all analytes across the measured ranges. The measured LLOQs were well below the therapeutic levels of these drugs, and the upper limits of quantitation (ULOQs) were well above minimum inhibitory concentrations of affected fungal pathogens (7, 39–42). Additionally, the paper spray MS/MS assay ranges were similar to ranges reported by commercial reference laboratories, such as Mayo Clinic Laboratories and ARUP laboratories.

Typically, the acceptance criteria is 15 – 20% for validated methods depending on the concentration of the QCs (34). On average, the overall precision (%CV) and accuracy (%bias) were ≤20% highlighting the quantitative capabilities of this method. The external QC further reinforced the accuracy of the method as they were prepared by a commercial external source; neither the %CV nor the %bias for the external QC material exceeded 15%. An acceptance criteria of ≤25% was chosen for posaconazole to accommodate the greater variation arising from the lack of an isotopically-labeled analog for this analyte. A commercially available, stable isotopically-labeled analog of posaconazole (posaconazole-D4) was evaluated. However, posaconazole-D4 has the same nominal mass as itraconazole, and interference on the itraconazole SRM channels arising from posaconazole-D4 was observed.

Matrix effects are often evaluated by comparing analyte response in neat solution to analyte response in biological samples. This approach is not particularly informative for this assay due to the use of internal standardization and matrix-matched calibrants. Instead, matrix effects were evaluated by determining the variability of calibration slopes generated in multiple different plasma donors. This approach addresses the most relevant question about the effect of matrix on analytical measurements (38): Can a calibration curve generated in a single lot of biofluid be used to determine analyte concentrations in different lots? The variation in slopes was ≤4% for all analytes except posaconazole, which had a higher variation (10%) due to the lack of an isotopically-labeled internal standard. This variation arising from matrix was deemed insignificant.

Remnant clinical samples were analyzed by the paper spray MS/MS method and a validated external reference laboratory LC-MS/MS assay to further assess correlation and agreement between the two methods. The Kolmogorov-Smirnov CUSUM test of normality indicated that the relationship was linear (p = ≥0.5450). Correlation was further assessed via Passing-Bablok regression, and it demonstrated good linearity throughout the measured range (Figure 3). However, the regression analysis showed there was a statistically significant underestimation for itraconazole and hydroxyitraconazole by the paper spray MS/MS method. The Bland-Altman plots also showed a statistically significant systematic underestimation for all analytes (Figure 3). Across all of the analytes, ~95% of the data points did lie within the limits of agreement (±1.96*SD), indicating the relative differences were normally distributed (43). While the bias could have arisen due to the method itself, many other factors could have also caused these discrepancies. There were systematic differences in sample age, handling, transport, and storage conditions, as well as different calibration materials used for the two assays. Inter-laboratory variation has been widely reported in the literature for many analytes, including triazoles. At present, we identified only one study assessing the agreement between triazole mass spectrometry-based

quantitative assays utilized by different laboratories (44). In that work, voriconazole measurements made by two different HPLC-MS methods at different laboratories had an average difference of 4%, which is comparable to what we obtained for paper spray MS. Several studies assessing agreement between quantitative immunoassays and mass spectrometry- or chromatographic-based methods have been conducted, and wide variations has been reported (45–48). Furthermore, there have been reports of internal standard choice affecting triazole quantitative results (47, 49).

From a clinical standpoint, the underestimation obtained here would not alter patient management. For example, the therapeutic trough range for voriconazole is 1.0 – 5.5 μg/mL (7, 14), and a trough of <1.0 μg/mL would prompt the clinician to increase the dose for efficacy. In the voriconazole clinical specimens with drug levels <1.0 μg/mL as measured by LC-MS/MS, the relative difference between the two methods ranged from −34% to +24%. This equates to absolute differences of −0.17 to +0.13 μg/mL, which is negligible. In terms of safety, voriconazole toxicity (i.e. neurotoxicity, self-limited photopsia, hepatotoxicity) is typically seen at >5.5 μg/mL (7, 50). It is unlikely that toxicity would be exacerbated due to drug level underestimation as the relative differences between the two methods' results were much smaller at concentrations of >4 μg/mL (−15% to 6%).

A similar argument can be made for itraconazole and posaconazole, which have therapeutic trough goals of >1.0 μg/mL and >0.7 μg/mL (>1.0 ug/mL for severe disease), respectively. In itraconazole clinical specimens <1.0 μg/mL by LC-MS/MS, the relative difference between the two methods ranged from −53% to −6% (absolute difference: −0.38 to −0.02 μg/mL), which indicates a negative bias for paper spray MS/MS. For posaconazole clinical specimens <1.0 μg/mL by LC-MS/MS, the negative bias was not as prominent with relative differences between the two methods ranging from −26% to −4%, or absolute differences of −0.2 to −0.04 μg/mL. As with voriconazole, the physician would be prompted to increase the dose of the triazole if levels did not meet therapeutic goals. For itraconazole, toxicity at high concentrations is less of a concern as absorption is problematic, and dosage is often limited by clinical side effects (i.e. gastrointestinal intolerance, fluid retention) rather than a maximum "toxic" plasma concentration. For posaconazole, absorption can also be difficult, and there is insufficient data to establish a maximum plasma concentration as there is no relationship between adverse effects and plasma concentrations. Therefore, while the negative bias for itraconazole and posaconazole are statistically significant, this issue is unlikely to be clinically significant.

Another important issue facing paper spray is interference, which could cause an over-estimation of the target compound due to the lack of separation. Endogenous interferences can be identified by analysis of drug-free plasma from several donors. Another source of interference are labile metabolites of the parent drugs that can fragment in-source to yield the parent drug. This type of interference was reported previously for posaconazole glucuronides (51). Our analysis of posaconazole glucuronide standard revealed an interference of 3%, which was deemed insignificant considering the plasma concentrations of the glucuronide metabolites are 3–4 times lower than posaconazole itself (52). The extent of interference will vary with different MS models; interference from glucuronide metabolites should therefore be re-evaluated for each type of mass spectrometer.

In conclusion, the developed paper spray MS/MS method agreed reasonably well when compared to the “gold” standard LC-MS/MS method. This study showed that rapid quantitation of triazoles utilizing paper spray MS/MS is feasible and may prove to be a powerful tool for clinical care and research. Further studies utilizing clinical specimens are needed to determine the cause of systematic underestimation of triazole quantitative results obtained with in-house paper spray MS/MS compared to send-out HPLC-MS/MS results. However, this issue is unlikely to have any significant clinical effect.

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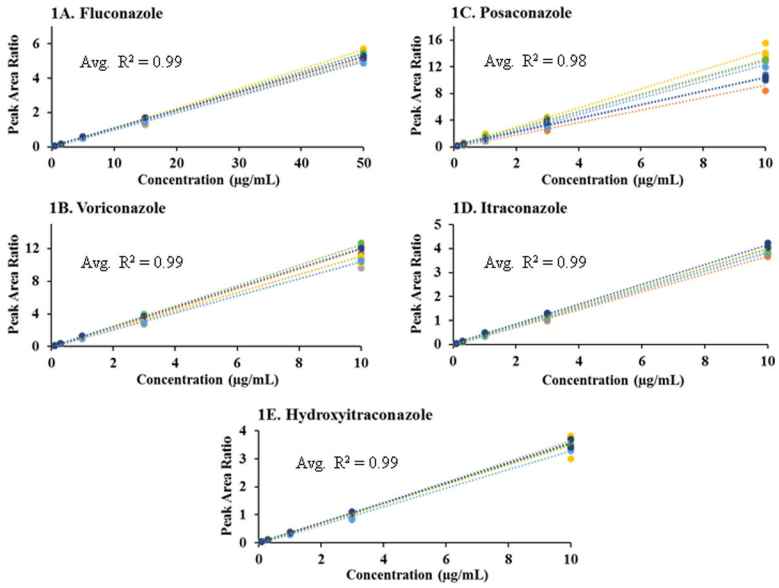

Figure 1:

Overlay of calibration curves for the five triazoles, fluconazole (**1A**), voriconazole (**1B**), posaconazole (**1C**), itraconazole (**1D**), and hydroxyitraconazole (**1E**), collected over seven different days (one calibration curve/day). Linearity ranged from R^2 = 0.94–0.99.

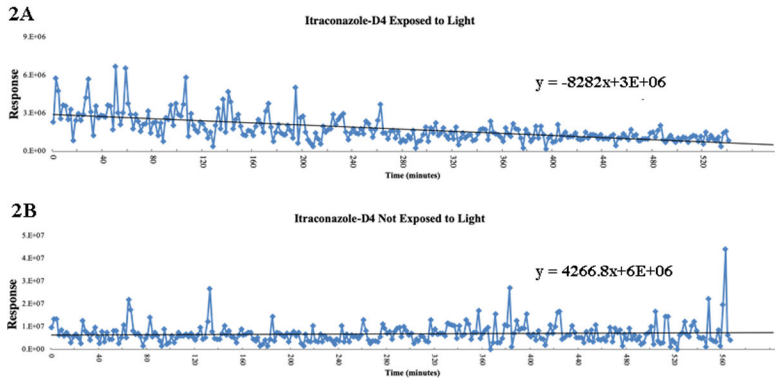

Figure 2:

Signal response of the stable isotopic internal standard, itraconazole-D4, in plasma samples stored under ambient light and plasma samples stored in a dark environment. There was a decrease in the internal standard signal response over the course of 16 hours (**2A**) when exposed to light. This problem was corrected by shielding the samples from ambient light (**2B**).

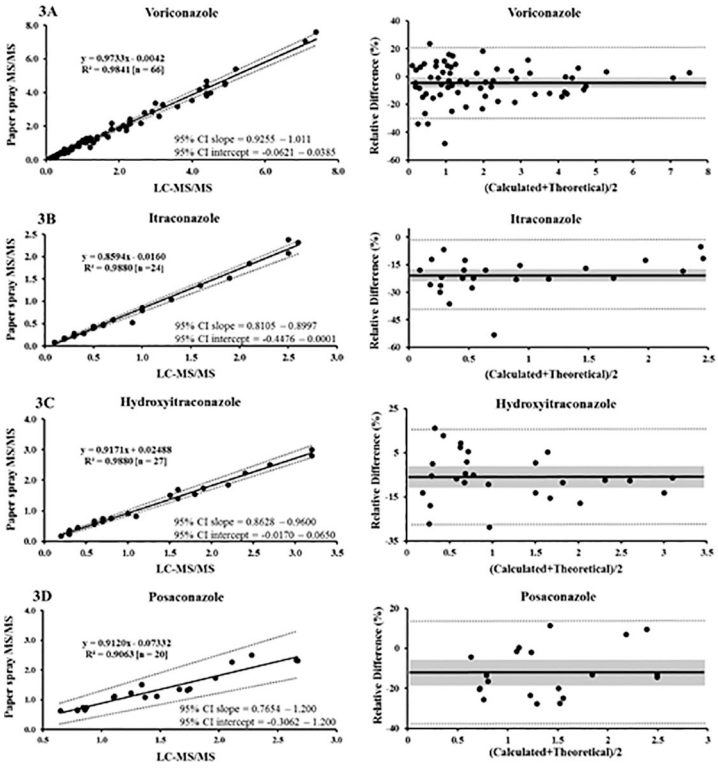

Figure 3:

Passing-Bablok regression (left) comparing paper spray MS/MS to the reference laboratory LC-MS/MS method. Dotted lines represent the upper and lower confidence intervals. Overall, the data showed good linearity (R^2 = 0.90–0.98). Bland-Altman plot (right) depicting the %relative differences between the paper spray MS/MS and LC-MS/MS reference value. The solid line indicates the mean of the %relative difference of the two methods. The shaded region depicts the 95% confidence intervals for that mean. The dotted line represents the limits of acceptance of +/−1.96*SD.

Table 1:

The analytes investigated, molecular formulas, parent ions, quantifying and confirming ions, S lens, and CE parameters.

Compound Name	Chemical Formula	Parent m/z	Fragment m/z*	S lens	CE (eV)
Voriconazole	$C_{16}H_{14}F_3N_5O$	350	**281**	83	17
			263		20
			224		18
Voriconazole-D3	$C_{16}H_{11}D_3F_3N_5O$	353	**284**	84	16
			266		27
			224		19
Fluconazole	$C_{13}H_{12}F_2N_6O$	307	**220**	82	18
			238		16
			169		23
Fluconazole-13C3	$C_{10}[13]C_3\ H_{12}F_2N_6O$	310	**223**	82	18
			241		16
			172		24
Itraconazole	$C_{35}H_{38}Cl_2N_8O_4$	705	**392**	169	35
			348		39
			335		40
Itraconazole-D4	$C_{35}H_{34}D_4Cl_2N_8O_4$	709	**396**	162	35
			352		39
			339		43
Hydroxyitraconazole	$C_{35}H_{38}Cl_2N_8O_5$	721	**408**	179	45
			392		39
			173		57
Hydroxyitraconazole-D5	$C_{35}H_{33}D_5Cl_2N_8O_5$	726	**413**	211	37
			397		33
			255		41
Posaconazole	$C_{37}H_{42}F_2N_8O_4$	701	**344**	174	44
			370		41
			614		32

*Bold items indicate the quantifier ion for each analyte

Table 2:

The average coefficient of determination (R^2), average relative error of the slope (%), average LOD (μg/mL), and measured LLOQ values (μg/mL) for the five triazoles collected over seven runs on seven separate days. The standard deviation of the calculated LOD is also shown.

Target	Average R^2	Average Relative Error of Slope (%)	Average LOD* (μg/mL)	Measured LLOQ≠ (μg/mL)
Fluconazole	0.99	2	0.06 +/− 0.02	0.5
Itraconazole	0.99	2	0.01 +/− 0.00	0.1
Posaconazole	0.98	5	0.03 +/− 0.01	0.1
Voriconazole	0.99	2	0.02 +/−0.01	0.1
Hydroxyitraconazole	0.99	3	0.02 + 0.01	0.1

LOD = 3(standard error of the intercept/slope)

≠LLOQ = Concentration at which the signal-noise ratio was consistently ≥10

Table 3

: Accuracy (%bias) and precision (%CV) were calculated across seven experimental days. The intra-day %bias and %CV values are the average value obtained within a run across all seven days. The inter-day %bias and %CV were calculated for every replicate across all seven days.

	Intra-day accuracy* and precision≠									
	QC LLOQ		QC Low		QC Medium		QC High		QC External	
Analyte:	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)
Fluconazole	1	−5	8	−7	−1	−4	1	−4	−2	−2
Hydroxyitraconazole	11	−13	8	−7	3	−6	7	−6	8	−7
Itraconazole	3	−7	5	−7	−1	−3	−3	−4	−10	−2
Posaconazole	1	−12	2	−15	−4	−10	−6	−10	−11	−8
Voriconazole	−16	−2	3	−2	−8	−6	−6	−6	−1	−4
	Inter-day accuracy* and precision≠									
	QC LLOQ		**QC Low**		**QC Medium**		**QC High**		**QC External**	
Analyte:	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)
Fluconazole	−2	−10	8	−10	−1	−5	1	−6	−2	−4
Hydroxyitraconazole	10	−16	8	−10	3	−8	8	−9	11	−11
Itraconazole	1	−13	5	−11	−1	−5	−2	−6	−10	−6
Posaconazole	−2	−17	0	−18	3	−10	−5	−15	−12	−13
Voriconazole	−9	−13	1	−19	−7	−7	−5	−10	−2	−7

*%Bias = (Grand mean of calculated concentration-nominal concentration/nominal concentration)*100

≠%CV = (Standard deviation/mean)*100